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PRINCIPAL INVESTIGATOR: Michael C. Archer, Ph.D.


CONTRACTING ORGANIZATION: University of Toronto
Toronto, Ontario, Canada M5S 1A1


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**6. AUTHOR(S)**
Michael C. Archer, Ph.D.

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

University of Toronto
Toronto, Ontario, Canada  M5S 1A1

E-Mail: m.archer@utoronto.ca

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The central hypothesis we are addressing is that inhibition of mammary carcinogenesis by n-3 polyunsaturated fatty acids (PUFAs) can be accounted for by their inhibitory effect on the cholesterol biosynthesis (mevalonate) pathway. In Task 3, we have shown that the n-3 PUFA docosahexaenoic acid (DHA) inhibits MCF-7 cell proliferation in part though inhibition of mevalonate synthesis. Mechanisms other than inhibition of mevalonate synthesis, however, appear to be responsible for the inhibitory effects of eicosapentaenoic acid (EPA) on growth of MCF-7 cells. In Task 4 (new), we determined that mevalonate promotes the growth of mammary tumors in nude mice, and of human breast cancer cells in culture. This effect is associated with alterations in G1 regulatory proteins that support initiation of DNA synthesis. This finding has major significance for both prevention and treatment of mammary cancer, since mammary mevalonate synthesis may be increased by common treatments that lower serum cholesterol levels (e.g. use of bile acid sequesterants and statins). Our work will provide a basis for understanding the protective effects of n-3 PUFAs and perhaps other dietary factors on breast cancer development and may lead to mechanism-based strategies for the prevention of breast cancer.

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# INTRODUCTION

Disregulation of the mevalonate pathway is believed to contribute to uncontrolled tumor cell growth [1,2]. Malignant cells from diverse tumor types [3-5] including mammary tumors [2] have elevated mevalonate synthesis due to increased levels and activity of 3-hydroxy-3-methyl glutaryl Coenzyme A reductase (HMG-CoA reductase). Excess mevalonate provided to cells in culture can stimulate entry into the cell cycle, and promote proliferation [6,7]. Conversely, reduced mevalonate synthesis results in improved growth control, and severe mevalonate depletion causes a G1 phase growth arrest. Mevalonate can be limiting for DNA synthesis and cell cycle progression [8]. Inhibition of HMG-CoA reductase activity by statin drugs slows proliferation of both normal [1,9-11] and neoplastic cultured cells [10,12-14]. This is associated with differentiation [15,16], and apoptosis [17,18]. Similar effects are also seen *in vivo* as HMG-CoA reductase inhibitors impair tumor growth in rodent models [19-22]. Corresponding to this drug effect, a number of dietary compounds have been postulated to influence mammary tumorigenesis by modulating mevalonate synthesis (e.g. fish oils [23], cholesterol [24], plant isoprenoids [25-29], and linoleic acid [23]). Among these, fish oils have been found to both inhibit rodent mammary tumorigenesis [30-32], and rodent mammary HMG-CoA reductase activity [23]. However, while this correlation is indeed interesting, causality has not yet been established. In fact, although mevalonate appears convincingly to be of importance for the growth of transformed cells, the direct effects of mevalonate on mammary tumorigenesis *in vivo* are unreported. We hypothesized that mevalonate is limiting for the growth of mammary tumors *in vivo,* and for cell cycle progression *in vitro*. Inhibition of HMG-CoA reductase activity by fish oils, therefore, would impair cell proliferation due to reduced cellular mevalonate levels. The objective of this research is to test this hypothesis in cell culture and rodent models, and examine whether a similar mechanism could be operative in human breast cancer development. Tasks 1 and 2 are completed and were reported on last year. Task 3 is reported below, along with additional work performed on the role of mevalonate in human breast cancer cell cycle control, which is reported as Task 4 (new).

# BODY

**Task 3**   To determine the extent to which growth inhibition of MCF-7 cells by n-3 polyunsaturated fatty acids (PUFAs) is reversed by mevalonate.

In initial experiments we have optimized cell culture conditions. The issue of ensuring that the lipophilic fatty acids are adequately solubilized in aqueous media has prompted us to experiment with varying culture conditions, including different levels of ethanol (up to 1%) and albumin (up to 2g/L). Reported conditions in the literature vary considerably, even for commonly used cell lines, and so we wanted to ensure that culture conditions achieved optimal solubilization of fatty acids while maintaining favorable growth conditions and minimizing toxicity of vehicles used. Our protocol involved extensive mixing and pre-incubation of fatty acids with albumin (final concentration in media 2g/L) to allow for maximal binding to the protein carrier. Final ethanol concentrations were minimized, with a maximum concentration in medium of 0.05%. Low-serum medium (2% fetal bovine serum) was used to support MCF-7 growth during test treatments.

The effects of the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on MCF-7 cell proliferation were assessed. Cells grown in 96 well plates were treated for 11 days with increasing levels of the test fatty acids (10, 30, 50, and 70µM). Cell proliferation rates were determined by BrdU incorporation as measured by ELISA. Both fatty acids inhibited cell proliferation in a dose-dependent manner (Figs. 1a & 1b). Similar effects were seen on HMG-CoA reductase activity. Both EPA and DHA inhibited HMG-CoA reductase activity in MCF-7 cells grown for 8 days in 75 dL flasks (Figs. 2a & 2b). The maximum effect was seen at 30uM for both fatty acids, and there was no greater inhibition of HMG-CoA reductase activity above this level. Inhibition of HMG-CoA reductase activity by fish oils was highly correlated with inhibition of cell proliferation (Figs. 3a & 3b). We next assessed the ability of mevalonate to restore proliferation in cells treated with EPA or DHA. Cells grown in the presence of 30uM EPA or DHA (the level at which maximum effects on HMG-CoA reductase were observed) were treated with increasing levels of mevalonate, up to 1mM. Mevalonate increased proliferation in DHA treated cells by up to ~30% over cells grown in the absence of exogenous mevalonate (Fig. 4). EPA treated cells failed to respond to treatment with mevalonate, even at the highest (1mM) treatment level. Treatment of cells with 1mM mevalonate alone (in the absence of n-3 PUFAs) did not significantly promote proliferation. We concluded from this work that inhibition of HMG-CoA reductase activity accounts for some, but not all, of the inhibitory effect of DHA on MCF-7 cells. However, mechanisms other than inhibition of mevalonate synthesis appear to be responsible for most, if not all, of the inhibitory effects of EPA on MCF-7 cell growth.

**Task 4 (new).** To investigate a mechanism whereby mevalonate may promote mammary tumor cell growth.

We reported last year the results from a study in which we determined that mevalonate promotes the growth in nude mice of tumors derived from an inoculation of MDA-MB-435 human breast cancer cells (Figs. 5 & 6). While inhibition of mevalonate synthesis has consistently been associated with impaired growth of cells, the influence of excess mevalonate on mammary tumorigenesis is a novel and exciting finding. Mevalonate is a common cellular metabolite, and its synthesis is influenced by several factors including modulators of serum cholesterol levels such as bile acid sequesterants, statin drugs, and dietary compounds. The promoting role for mevalonate in mammary tumor growth may have wide ranging applications in the development of strategies for both the prevention and treatment of mammary cancer. To better understand the mechanisms underlying this promoting effect of mevalonate a series of experiments were performed on MDA-MB-435 human breast cancer cells in culture. In these experiments, we examined the effects of mevalonate on both cell proliferation and cell cycle control. Cell cycle progression is regulated in part by the activity of a group of kinases, known as cyclin-dependent kinases (CDKs) that are present at relatively constant levels during all phases of the cell cycle [33,34]. Their activity is modulated by the presence of two types of regulatory proteins [33]. The first are the cyclins, proteins that bind CDKs and form active cyclin-CDK complexes. In normal cells, levels of cyclins vary depending on cell cycle position and they control the ordered entry of cells into sequential phases of the cell cycle [34]. Because of the important role that they play in cell proliferation, cyclins are often over-expressed, or inappropriately expressed, in malignant cells. This allows cells to remain in cycle independent of signals to withdraw into G0 (resting) phase [34,35]. Indeed, the G1 cyclins D1 [36] and E [37] are oncogenic when expressed in the mammary epithelium of transgenic mice. The second group of regulatory proteins are CDK-inhibiting proteins (CDKIs) [33]. These are small molecular weight proteins that bind to and inhibit CDKs. CDKIs are powerful modulators of cell cycle progression - relative binding levels of CDKIs may actually play a greater role in determining CDK activity than cyclin abundance [38]. We have examined the effects of mevalonate on expression of regulators of G1 phase progression (cyclins D1 and E, $p21^{cip1}$, and $p27^{kip1}$, and CDK2 and

CDK4), and on relative binding of the CDKIs $p21^{cip1}$ and $p27^{kip1}$ to the late G1 phase kinase CDK2.

**Study 1**: The influence of exogenous mevalonate on proliferation of MDA-MB-435 cells was determined by BrdU incoporation into DNA. Under serum free conditions, cells were treated with increasing concentrations of mevalonate for 60 hours, and rates of cell proliferation relative to control were determined. Mevalonate stimulated cell proliferation in a dose-dependent manner up to the 1mM treatment level, above which no further increases in growth rate were apparent (Fig. 7). Treatment of cells with 1mM mevalonate was also associated with alterations in cell cycle distribution as assessed by FACS analysis. Mevalonate promoted passage of cells beyond the G1 restriction point into active DNA synthesis (S-phase) (Table 1). As passage through the restriction point commits cells to at least one cycle of replication, alterations in cell cycle distribution associated with mevalonate treatment likely account for increased cell proliferation rates.

**Study 2**: Cyclins were isolated from cells grown for 60 hours in serum free media containing 1mM mevalonate, and immunodetectable levels of cyclins D1 and E, $p21^{cip1}$, and $p27^{kip1}$, and CDK2 and CDK4 were determined. Western blot analysis shows that compared to controls (serum free medium without mevalonate), cells grown in the presence of mevalonate had significantly elevated levels of cyclin E (2-fold increase, $p<0.0001$) (Fig. 8a, Fig. 9), and significantly lower levels of $p21^{cip1}$ (~15% lower, $p<0.05$) (Fig. 8b, Fig. 9). Immunodetectable levels of $p27^{kip1}$, CDK2, CDK4, and cyclin D1 did not change with mevalonate treatment (Fig. 5). Immunoprecipitation of CDK2, followed by Western blot analysis of $p21^{cip1}$ and $p27^{kip1}$ revealed that treatment of cells with 1mM mevalonate decreased CDK2 binding of CDKIs by greater than 30%. This amount exceeds any changes seen in absolute levels of CDKIs, and so cannot be accounted for simply by a decrease in CDKI levels.

Taken together, these results from *in vitro* experiments performed on MDA-MB-435 human breast cancer cells indicate that mevalonate plays a direct role in modulating G1 phase progression. These effects are mediated through alterations in relative levels and interactions of protein regulators of cell cycle progression that enhance passage of cells from G1 to S phase.

## KEY RESEARCH ACCOMPLISHMENTS

• Inhibition of mevalonate synthesis was demonstrated to mediate, at least in part, the inhibitory effects of DHA on MCF-7 cell proliferation. This effect was shown to be highly specific since EPA did not inhibit MCF-7 cell proliferation though modulation of HMG-CoA reductase activity.

• Exogenous mevalonate was demonstrated to promote the growth of tumors derived from MDA-MB-435 human breast cancer cells in athymic nude mice.

• The effects of mevalonate on protein and enzyme regulators of G1 phase cell cycle progression were established, providing one possible mechanism to explain the promoting effects of mevalonate on mammary tumorigenesis.


## REPORTABLE OUTCOMES

1. Inhibition of mammary cancer cell proliferation by DHA is mediated, at least in part, by inhibition of mevalonate synthesis. This effect is not generalized to all n-3 PUFAs, although others (e.g. EPA) may display similar inhibitory effects on HMG-CoA reductase activity. This work is currently being duplicated, and we anticipate manuscript preparation to be complete by Fall 2002.

2. Mevalonate promotes the growth of tumors *in vivo*, and proliferation of mammary cancer cells *in vitro*. These effects are associated with alteration in regulators of cell cycle control that promote passage of cells beyond the G1 restriction point and, therefore, elicit the onset of DNA synthesis and cell proliferation. A manuscript reporting outcomes from this work is currently in preparation.

3. Effect of mevalonate on MDA-MB-435 breast cancer cell growth in nude mice. Seminar Presented, Federation of Societies for Experimental Biology and Medicine (FASEB) Conference, New Orleans, USA, April 2002.

4. The role of the mevalonate pathway in mediating EPA and DHA inhibition of MCF-7 cell proliferation. Poster Presented, International Society for the Study of Fatty Acids and Lipids (ISSFAL) Conference, Montreal, Canada, May 2002.

## CONCLUSIONS

In normal rodent mammary tissue, a diet rich in n-3 PUFAs inhibits HMG-CoA reductase activity, thereby limiting endogenous mevalonate synthesis compared to a diet rich in n-6 PUFAs. To investigate whether n-3 PUFAs inhibit breast tumor cell proliferation by inhibiting endogenous mevalonate synthesis, we performed cell culture work in which the ability of mevalonate to rescue inhibition of MCF-7 cell proliferation by n-3 PUFAs (EPA and DHA) was ascertained. Inhibition of mevalonate synthesis by DHA appears to be causally linked to inhibition of cell proliferation. Up to 30% of the inhibitory effect of DHA on MCF-7 cell proliferation could be accounted for by restricted mevalonate availability. This effect was specific to DHA. While EPA inhibited both cell proliferation and HMG-CoA reductase activity in a manner similar to that seen in cells treated with DHA, proliferation was not rescued by the addition of exogenous mevalonate. This indicates that the primary inhibitory effects of EPA are unrelated to effects on mevalonate synthesis.

While technical limitations precluded our use of transgenic and knockout mice in the chemical carcinogenesis studies we originally proposed, work involving the nude mouse-tumor cell inoculation model has been productive. In preliminary feasibility studies reported last year we determined that implanted mini-osmotic pumps could be used to deliver mevalonate to the mammary glands of mice, but not rats. This excluded the possibility of investigating the effects of mevalonate on rat mammary carcinogenesis, but allowed us to go ahead with our investigation of the effects of mevalonate on the growth of human breast cancer cells in mice. Tumors derived from highly malignant MDA-MB-435 human breast cancer cells grew more rapidly in nude mice implanted *s.c.* with mini-osmotic pumps containing mevalonate compared to controls, and had greater weights and volume at the end of the experiment (13 weeks). This indicates that growth of mammary tumors in this model is limited by the concentration of mevalonate, a result that supports our hypothesis that this metabolite plays a key role in controlling the rate of cell proliferation. Investigations involving MDA-MB-435 cells in culture have shed light on the specific nature of that role. Mevalonate promotes the proliferation of cells by increasing levels of the CDK2-enhancer protein cyclin E, while decreasing levels and/or CDK2 binding of the CDKIs $p21^{cip1}$ and $p27^{kip1}$.

Mevalonate, a key metabolite in all living cells, is required for the synthesis of cholesterol and hence membranes, and for the production of the isoprenoids that have growth regulatory functions. Indeed, mevalonate is required for DNA synthesis and cell proliferation. Several plant compounds, as well as fish oils, can inhibit mevalonate synthesis in cells. Successful completion of our experiments will provide a basis for understanding the protective effects of n-3 PUFAs and, perhaps, other dietary factors on breast cancer development and may lead to mechanism-based strategies for the prevention of the disease.

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(a)



(b)
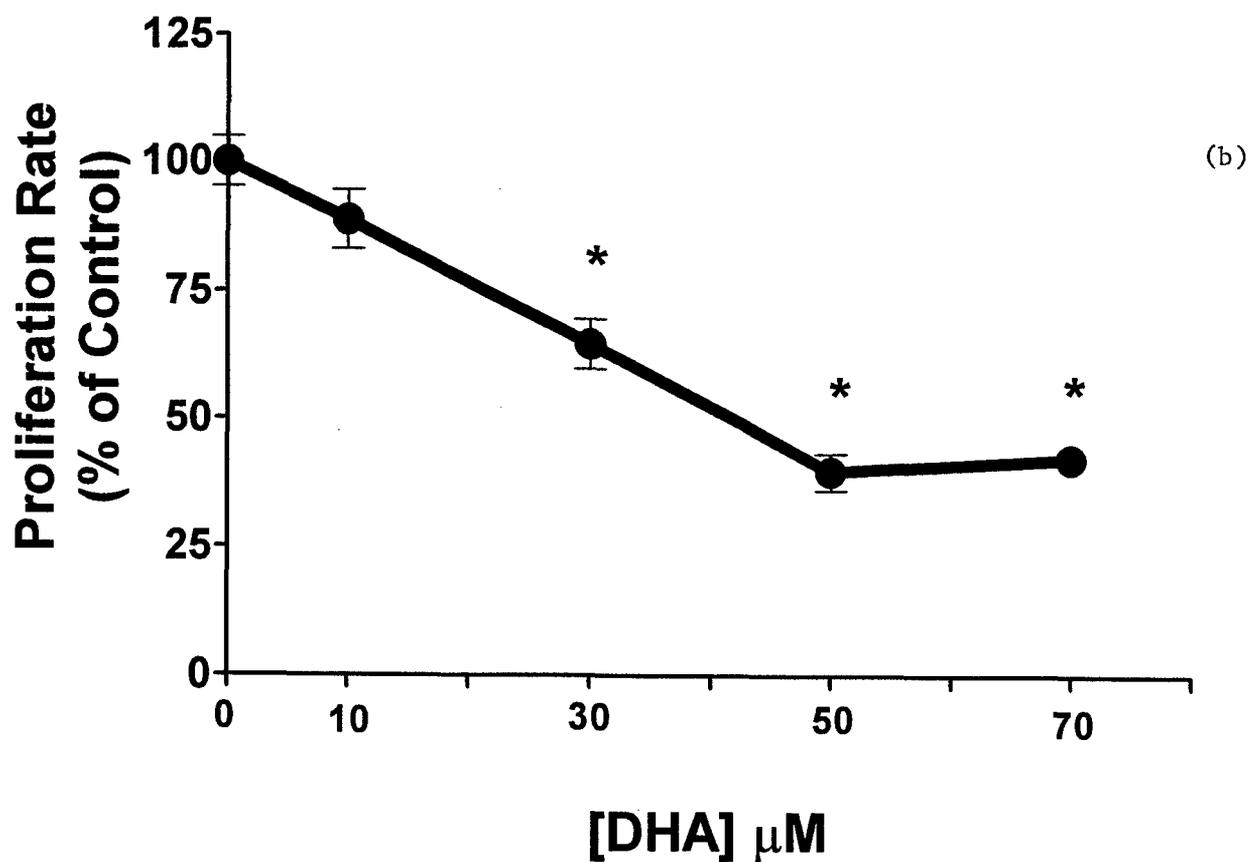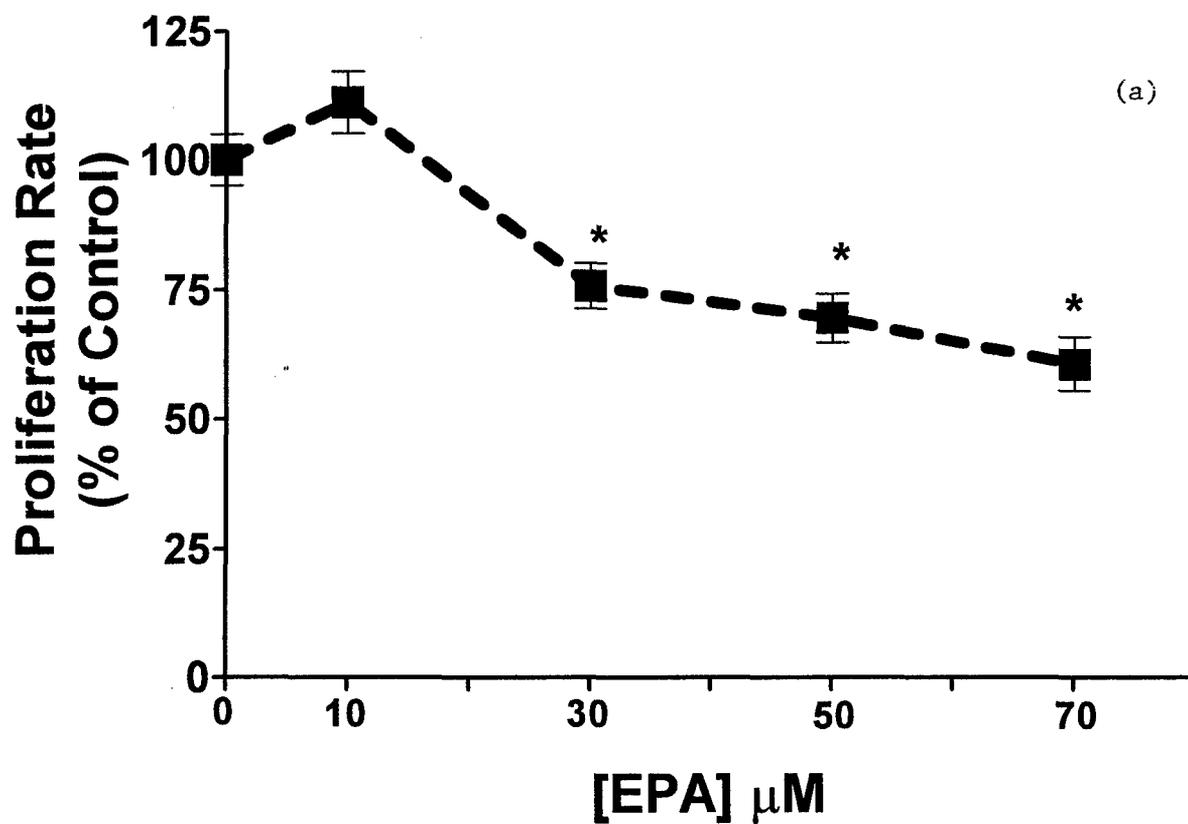
Figs. 1a & 1b. Effect of EPA and DHA on MCF-7 cell proliferation. Cells were treated with increasing levels of n-3 PUFAs as indicated for 11 days. Proliferation rates were assessed by ELISA assay of BrdU incorporation. Treatment of cells with fatty acids at levels of 30 µM or above significantly inhibited proliferation compared to controls (*p<0.01).

(a)



(b)
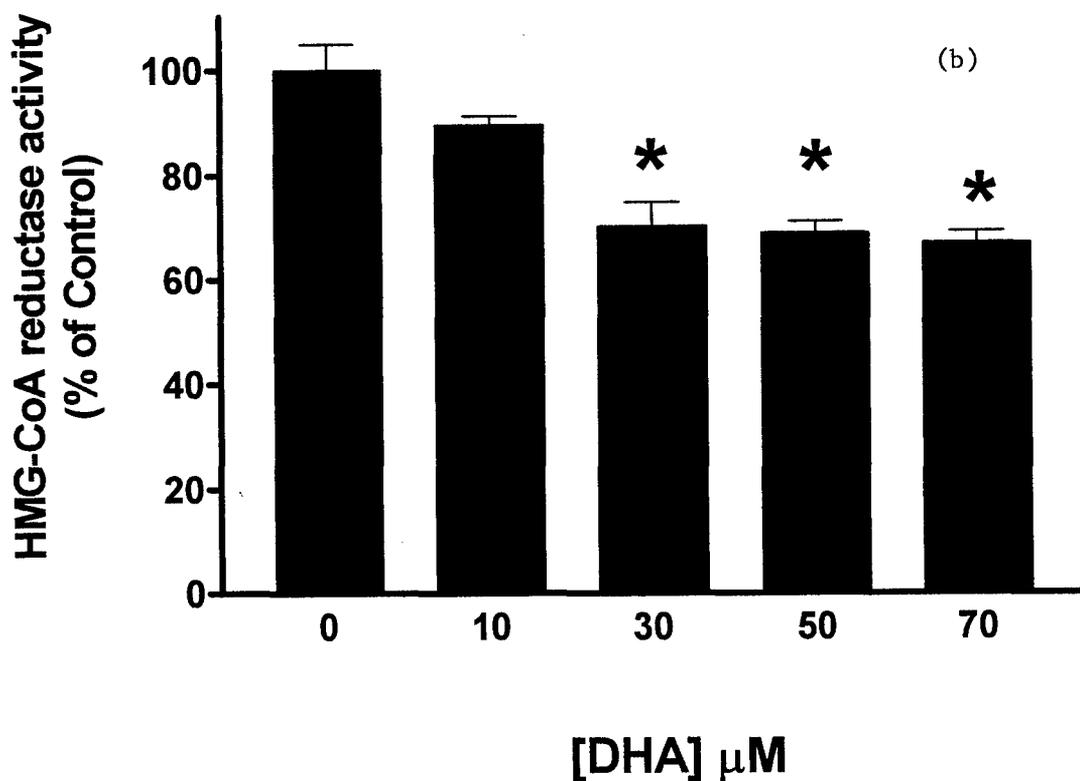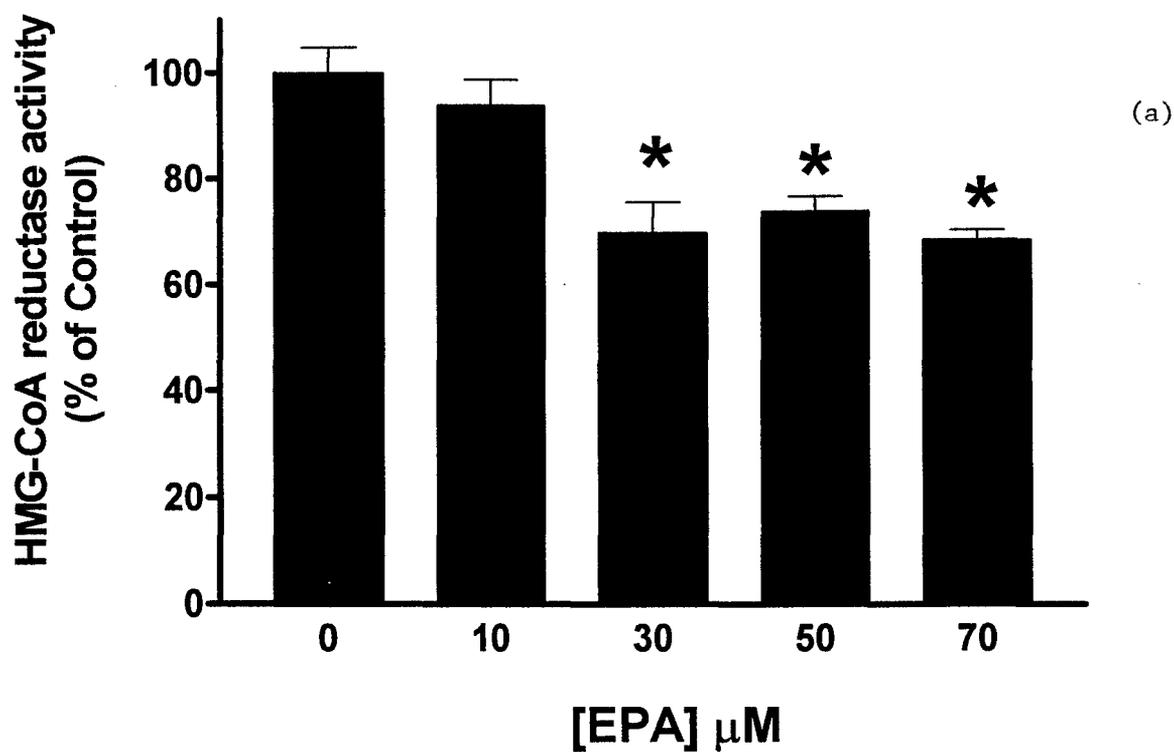
Figs. 2a & 2b. Effects of EPA and DHA on HMG-CoA reductase activity in MCF-7 cells. Cells were treated for 8 days. Data represent mean activity assayed from 3 separate flasks (*p<0.01 versus control).
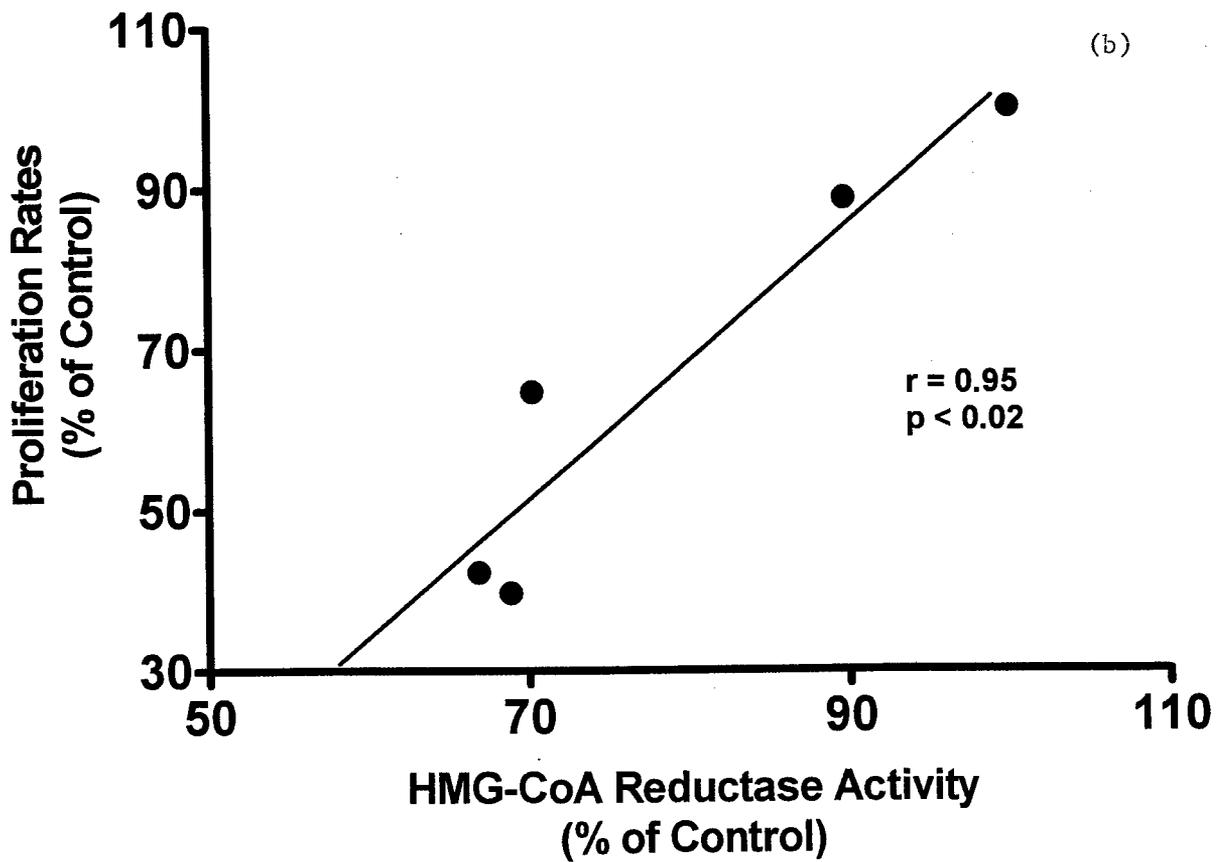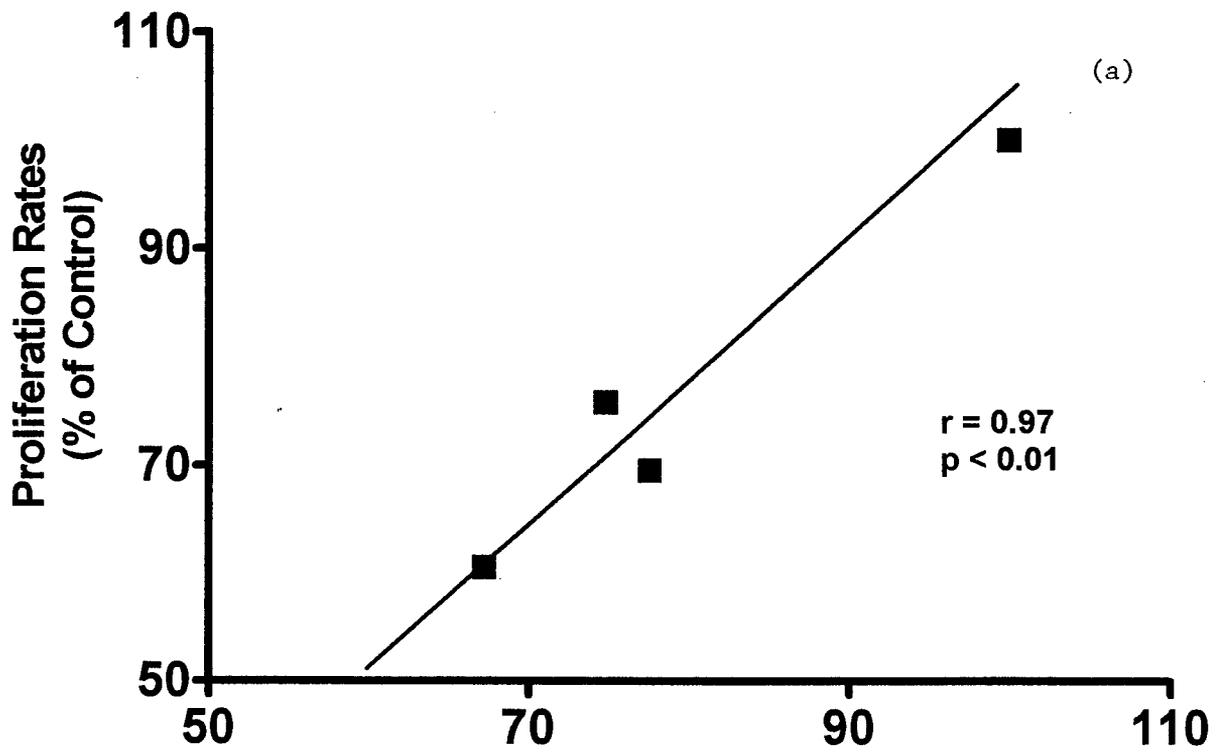
Figs. 3a & 3b. Correlation between cell proliferation rate and HMG-CoA reductase activity in MCF-7 cells treated with varying levels of EPA (a) or DHA (b).
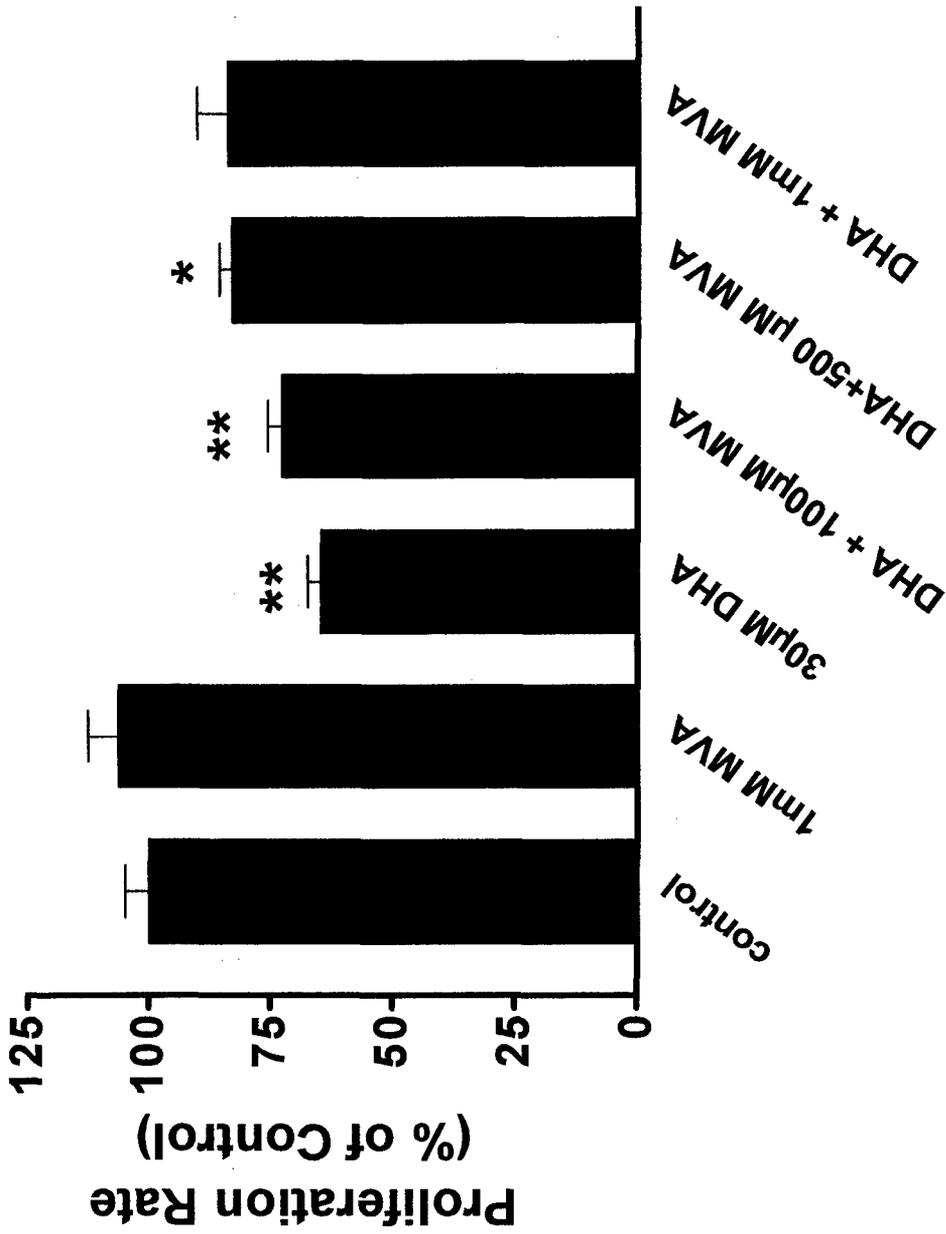
Fig. 4. Mevalonate partially restored cell proliferation in cells treated with 30 µM DHA (1mM mevalonate caused a 29.6 ± 9.7% increase in proliferation rate). 1mM mevalonate alone had no effect on cell proliferation. **p<0.01 vs control; *p<0.05 vs control).
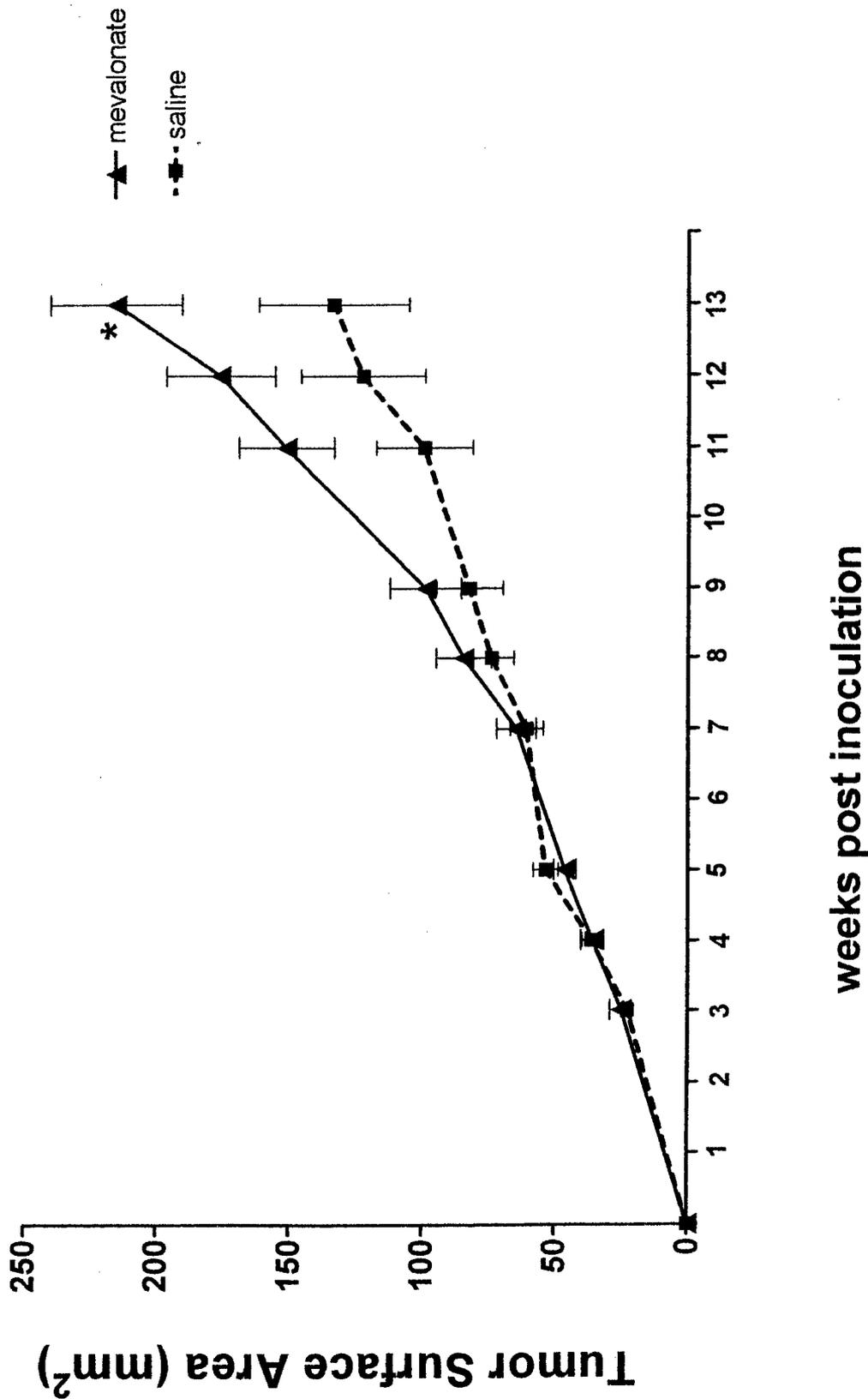
Fig. 5. The effect of mevalonate on the growth of tumors derived from an inoculation of $10^6$ MDA-MB-435 human breast cancer cells. Host nude mice were implanted with a mini-osmotic pump containing either mevalonate (1mg/μL) or isotonic saline (*p<0.05).
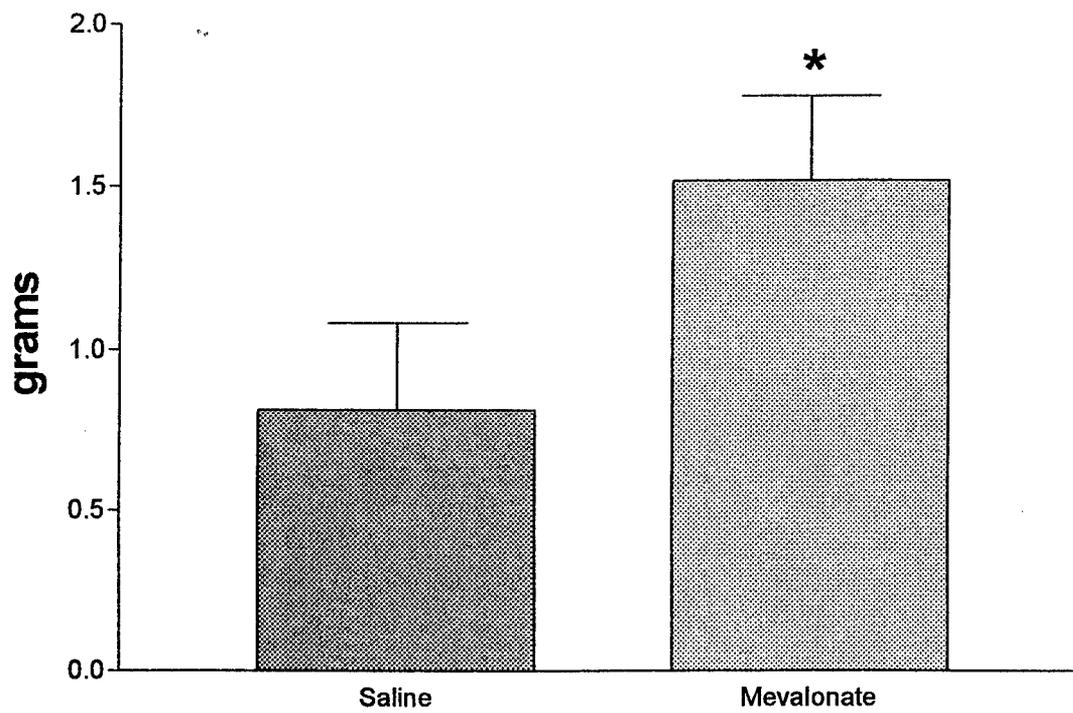
Fig. 6. Mean tumor weights at necropsy from mice implanted with mini-osmotic pumps containing either mevalonate or isotonic saline (*p<0.05).
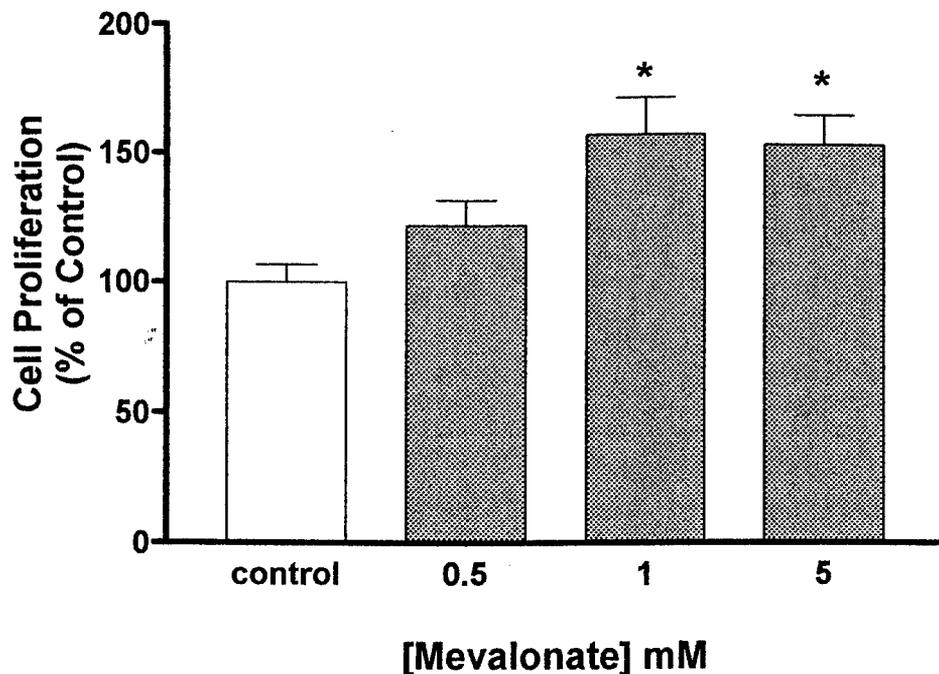
Fig. 7. Effects of mevalonate on growth of MDA-MB-435 cells in culture. Cells were grown for 60 hours in the presence of increasing levels of mevalonate, and proliferation rates were assessed by nuclear incorporation of BrdU (*p<0.01 vs control).

Table 1. Proportion of MDA-MB-435 cells in each phase of the cell cycle

| Phase | percentage of total cells | | |
| --- | --- | --- | --- |
| | Mevalonate[1] | Control | p |
| G0-G1 | 74 | 88 | 0.0007 |
| S | 18 | 6 | 0.0015 |
| G2-M | 8 | 6 | nsd |

[1] Cultures were given either 1mM mevalonate or $ddH_2O$ (control). Cells were grown for 60 hours and then harvested by trypsinization, and stained with PI for FACS analysis. Values represent a mean of 3 separately analyzed flasks.
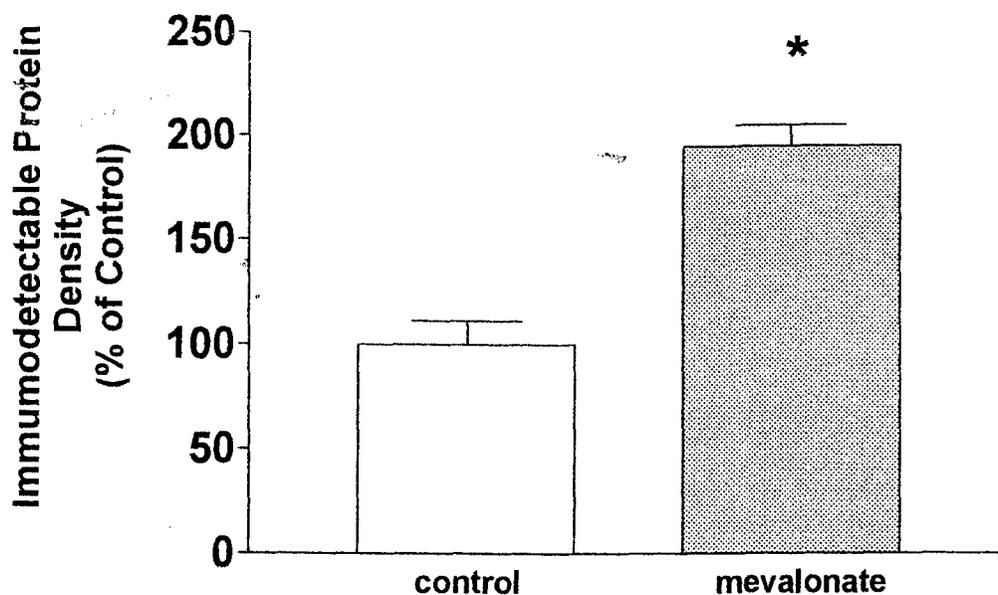
Fig. 8a. Immunodetectable levels of cyclin E were approximately 2-fold higher in homogenates of cells grown in the presence of 1mM mevalonate compared with controls (*p<0.0001). All cells were grown for 60 hours under serum free conditions.
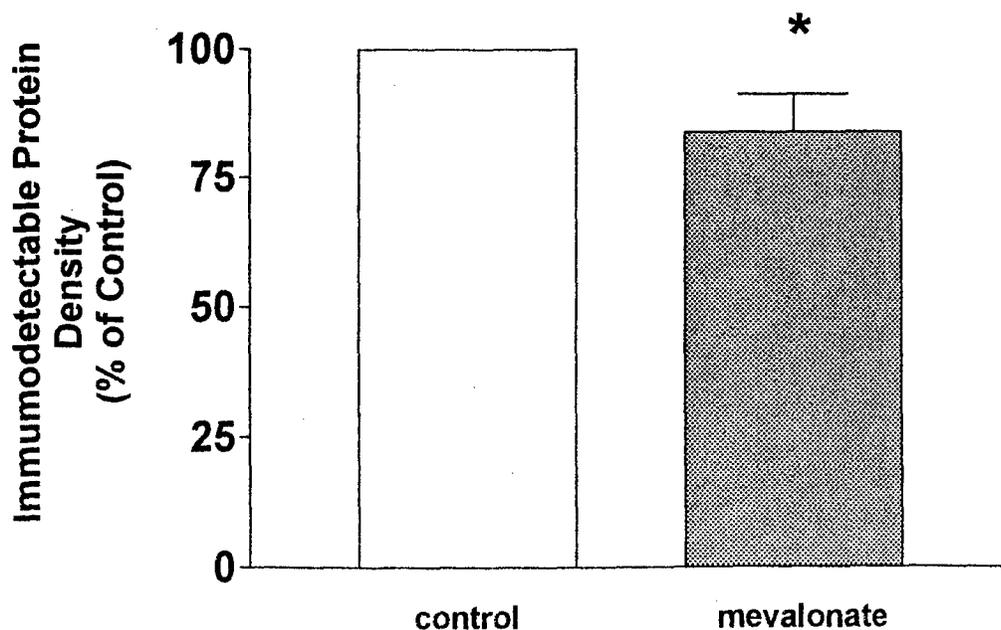


Fig. 8b. Immunodetectable levels of p21$^{cip1}$ were significantly lower in homogenates of cells grown in the presence of 1mM mevalonate compared with controls (*p<0.05).
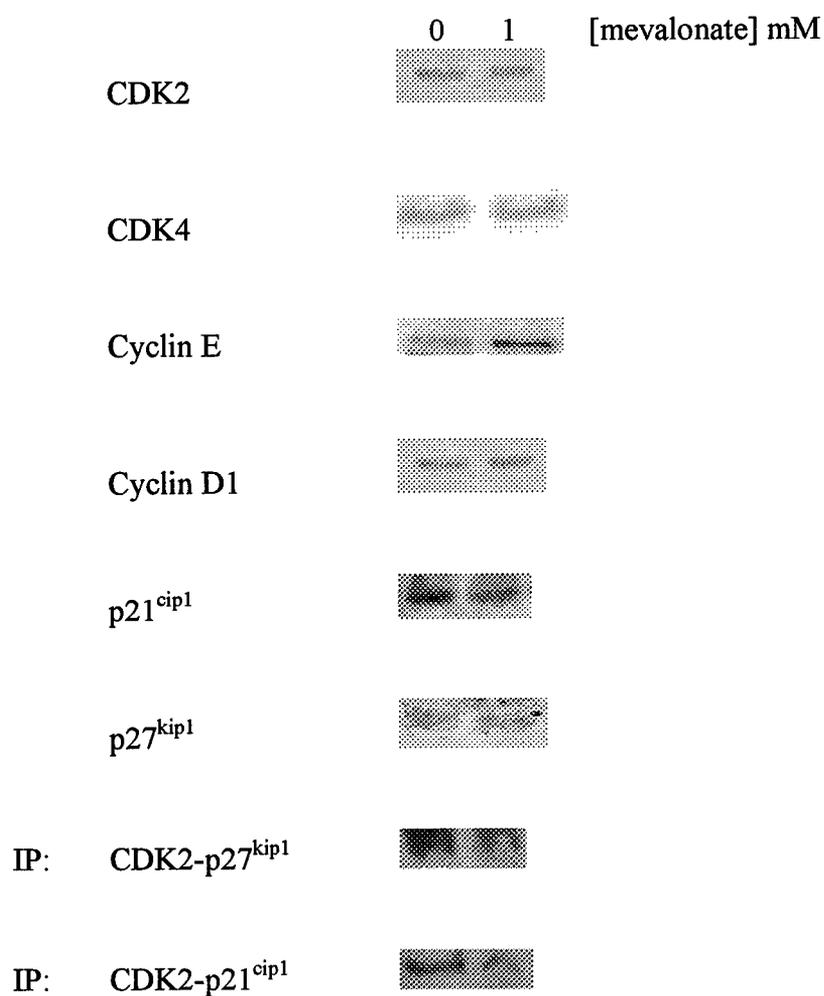
**Fig. 9. The effect of mevalonate on G1 phase regulatory proteins. Cells were treated for 60 hours in serum free medium either in the presence of mevalonate (1mM) or sterile H₂0 (control). Levels of indicated proteins were determined by Western blot analysis. Immumoprecipitation (IP) of cellular extracts with anti-CDK2 was performed, and immunoprecipitates were electrophoresed and probed with antibodies to p21$^{cip1}$ and p27$^{kip1}$ to determine relative binding. Figures shown are representative blots of samples analyzed from duplicate experiments.**